Leucocyte Typing V

White Cell Differentiation Antigens

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identical pattern of tyrosine phosphorylated polypeptides was observed. Prominent phosphorylated polypeptides were identified with M_r of 110, 72, and 40 kDa. Incubation of K-562 cells with MA73 (2ZC115) failed to induce a similar pattern of phosphorylated polypeptides. Incubation with the cross-linking antibody alone, GAM F(ab')$_2$, similarly did not induce novel phosphorylated polypeptides.

The pattern of tyrosine phosphorylation observed following cross-linking of anti-CD32 mAb is consistent with data previously reported utilizing Fab fragments of mAb IV.3 that showed that the 40-kDa tyrosine phosphorylated protein was FcγRII [8]. The identities of the other tyrosine phosphorylated substrates of M_r 110 and 72 kDa are currently under investigation.

The observation that mAb MA23 (BAS62-11) induced a similar pattern of tyrosine phosphorylated polypeptides but yet does not recognize CD32 suggests that this mAb binds an antigen on the surface of K-562 cells via the Fab domain and then activates FcγRII via its Fc region. This could be accomplished by: (1) formation of cellular immune complexes that could bind to FcγRII on other K-562 cells; or (2) tripartite engagement of IgG molecules on the same cell with subsequent cross-linking by the secondary antibody. This finding indicates that ascites containing whole immunoglobulin of an mAb directed against a different cell surface molecule could induce FcγRII-mediated tyrosine phosphorylation. Thus it points out the necessity of using Fab or F(ab')$_2$ fragments of mAb when investigating the cellular signal transduction mechanisms of any receptors on cells expressing FcγR. The physical cross-linking of such intact immunoglobulin molecules may produce patterns of tyrosine phosphorylated proteins similar to those induced by cross-linking of FcγRII alone.

Acknowledgement

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M8.2 Specificity of CD32 mAb for FcγRIIa, FcγRIIb1, and FcγRIIb2 expressed in transfected mouse B cells and BHK-21 cells

PETRA BUDDE, VOLKER WEINRICH, PETER SONDERMANN, NILS BEWARDER, ANDREAS KILIAN, OLAF SCHULZECK, and JÜRGEN FREY

Six monoclonal antibodies (mAb) of the CD32 panel were analysed for their specificity against the various FcγRII isoforms expressed in the FcγR− mouse B-cell line IIA1.6 [1] and BHK-21 cells [2]. In addition, we compared the reactivity of the mAb with the respective receptors homologously expressed in the human B-cell line Daudi (FcγRIIb1+ and FcγRIIb2+) as well as K-562 cells (FcγRIIaHR+/LR+; HR = high responder and LR = low responder). Besides the six Workshop antibodies, we included three new mAb obtained in our laboratory, 1A4, II1A5, and II8D2, which were compared with an mAb MA179 (AT10) known to recognize all CD32 isoforms [3].

Using FACS analysis, we found that the two mAb MA23 (BAS62-11) and MA73 (2ZC115) did not react with any of the FcγRII, independent of the cell lines

Table 1 Reactivity of mAb with homologously and heterologously expressed CD32 isotypes

CD32 isoforms[†]	Reactivity of mAb[*] MA179 (AT10)	MR7 (IV.3)	MA23 (BAS62-11)	MA72 (KB61)	MA73 (2ZC115)	MA128 (FL18.26)	MA126 (CIKM5)	1A4	II1A5	II8D2
IIA1.6 cell line										
FcγRIIaLR	+ + +	+ +	0	+ +	0	+	+ +	0	0	0
FcγRIIaHR	+ + +	+ +	0	+ +	0	+	+	0	0	0
FcγRIIb1	+ +	0	0	+ + +	0	+	0	0	0	0
FcγRIIb2	+ +	0	0	+ + +	0	+	0	0	0	0
BHK-21 cell line										
FcγRIIaLR	+ + +	+ +	0	+ + +	0	+ +	+ + +	0	+ +	+
FcγRIIaHR	+ + +	+ +	0	+ + +	0	+ +	+ +	0	+	ND
FcγRIIb1	+ + +	+ +	0	+ + +	0	+ +	+ +	0	+ +	+ +
FcγRIIb2	+ + +	+ +	0	+ + +	0	+ +	+ +	0	+ +	+ +
Other cell lines										
Daudi	+ + +	(±)	0	+ + +	0	+ +	+	+ +	0	0
K-562	+ + +	+ + +	0	+ + +	0	+ +	+ + +	0	0	0

[*]The FcγRII isoforms were transfected into FcγR − mouse B-cell line IIA1.6 and BHK-21 cells as described [1]. mAb were incubated with stable clones expressing the respective isoforms under saturating conditions.

[†]Bound mAb were detected using fluorescein isothiocyanate (FITC)-labelled $F(ab')_2$ fragment of goat anti-mouse IgG + IgM. Background fluorescence was detected using the respective mouse isotype controls (IgG/IgM) and the FcR-parental cell lines. 0, No reactivity; ±, very weak reactivity; + to + + + indicate increasing levels of reactivity; ND, not done.

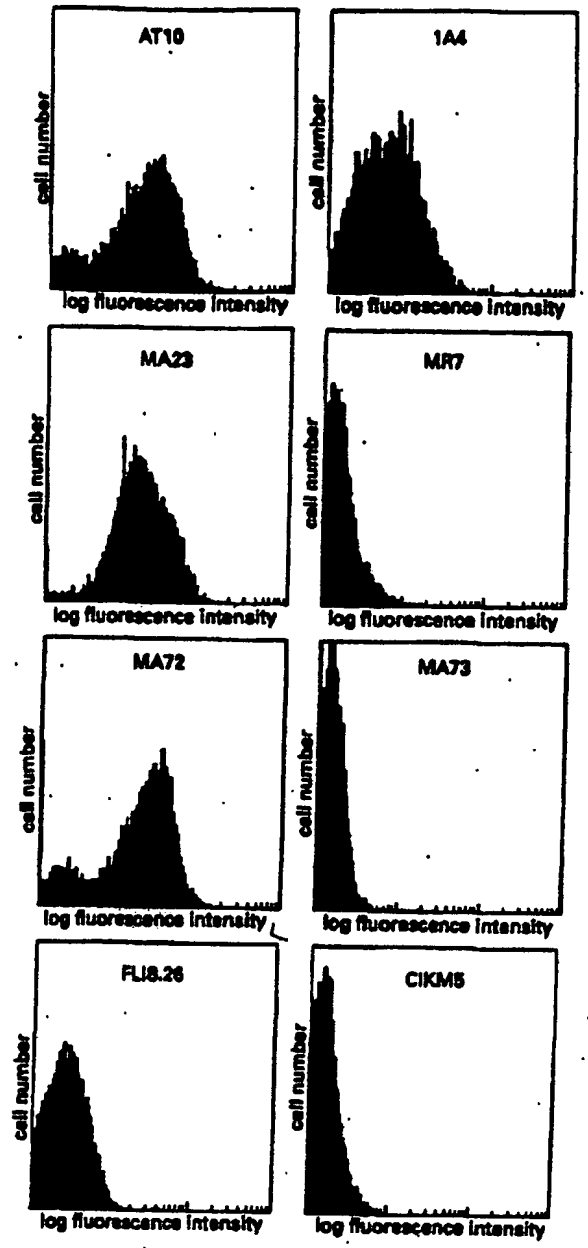

Fig. 1 Reactivity of CD32 mAb on CD19+ human B cells prepared from tonsils. After T-cell rosetting 98 per cent of the cells were CD19+. Cells (8×10^5) were incubated with the various Workshop mAb (1:100 diluted) and mAb AT10 and 1A4 (FcγRIIb-specific) as culture supernatants, followed by incubation with a fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG+IgM $F(ab')_2$ fragment and analysed by flow cytometry.

studied (Table 1). mAb MA128 (FLI8.26) recognizes FcγRIIa, FcγRIIb1, and FcγRIIb2 equally well, independently of the cell line studied. In contrast, mAb MA72 (KB61) shows a preferential binding to FcγRIIb1 and FcγRIIb2 in IIA1.6 cells (Table 1). mAb MR7 (IV.3) and MA126 (CIKM5) showed a strong preferential binding to FcγRIIa compared to FcγRIIb1 and FcγRIIb2, when expressed either in mouse or human B cells. Interestingly, this could only be observed in mouse B cells (IIA1.6) but not in BHK-21 cells. In this cell line the FcγRIIb isoforms are also recognized by MR7 (IV.3) and MA126 (CIKM5). Therefore, either different glycosylation patterns of the respective FcγRIIb isoforms or associated surface molecules in B cells are responsible for the varying antibody specificity. None of the mAb reacted with CD16–FcγRIIa chimeric receptors containing either 23 or 47 amino acids (aa) of the extracellular region of FcγRIIa (plus transmembrane and cytoplasmic region) [2]. Among the Workshop antibodies tested on human tonsillar B cells, only mAb MA72 (KB61) and MA128 (FLI8.26) gave positive results (Fig. 1). In contrast to all transfected cell lines analysed as well as Daudi and K-562 cells, MA23 (BAS62-11) gave bright fluorescence signals on human tonsillar B cells (Fig. 1).

Using a synthetic peptide (aa 30–39 of the mature protein) of FcγRIIb2 as well as FcγRIIb2 expressed in *Escherichia coli* we raised a panel of mAb with varying specificity. mAb 1A4 (IgM) directed against the synthetic peptide shows a strong specificity for FcγRIIb expressed in human B cells and B-cell lines comparable to that of mAb MA179 (AT10) and MA72 (KB61) (Fig. 1; Table 1). Interestingly, this mAb does not react with FcγRIIb1 and FcγRIIb2 expressed in mouse B cells (IIA1.6) as well as in BHK-21 cells (Table 1). Further studies (not described) revealed, that mAb 1A4 mostly reacts with activated B cells. The specificity of the antibody was verified by immunoprecipitation of FcγRIIb1 and FcγRIIb2 from Daudi cells (Table 2). The mAb II1A5 and II8D2 were raised against the FcγRIIb2 expressed in *E. coli* and were selected on BHK-21 cells expressing FcγRIIb2. In FACS analyses mAb II1A5 and II8D2 recognize FcγRIIa and FcγRIIb isoforms only when they are expressed on BHK-21 cells. In contrast, in Western blot analyses both antibodies detected FcγRII, independently of the cell line expressing the receptors (Table 2). Here, mAb II8D2 shows specificity for the FcγRIIb isoforms, whereas mAb II1A5 recognizes both FcγRIIa and FcγRIIb. Thus, it is possible to differentiate between FcγRIIa and FcγRIIb isoforms expressed in different cells and cell lines by Western blot analysis.

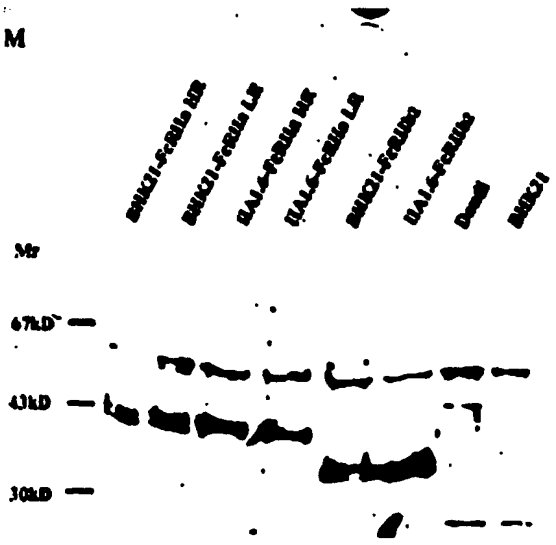

Fig. 2 Immunoprecipitation of homologously and heterologously expressed CD32 using mAb FL18.26. Immunoprecipitation and detection were performed as described in the legend to Table 2. The figure shows a representative result of the precipitation experiments summarized in Table 2.

The efficiency of the Workshop antibodies for immunoprecipitation of FcγRII isoforms from different cells was analysed using transfected BHK-21 and IIA1.6 cells as well as Daudi cells. The FcγRII precipitation was judged by immunoblotting using the new mAb IIIA5. Among the antibodies tested, only mAb AT10 and FL18.26 were able to bind both FcγRIIa and FcγRIIb isoforms with affinities sufficient to isolate the immune complexes (Table 2). These results confirm the data obtained by FACS analysis (Table 1). Using mAb MR7 (IV.3) we could only isolate the FcγRIIa from BHK-21 and IIA1.6 cells. The reactivity of MR7 (IV.3) against the FcγRIIb isoforms expressed in BHK-21 cells observed by FACS analysis (Table 1) must be a fairly weak binding because we could not isolate these FcγRII by immunoprecipitation (Table 2). Comparable results were obtained with mAb CIKM5. The only difference is that CIKM5 is more efficient in immunoprecipitating the FcγRIIaLR alloform (Table 2). The counterpart to MR7 (IV.3) and MA126 (CIKM5) for immunoprecipitation is mAb MA72 (KB61), which specifically reacts with the FcγRIIb isoforms (Table 2). This differential reactivity is not

Table 2 Immunoprecipitation efficiency of anti-CD32 mAb with homologously and heterologously expressed receptor isoforms*

mAb		Immunoprecipitation efficiency with†						
		BHK-21		IIA1.6		BHK-21	IIA1.6	Daudi
Workshop code	Clone name	FcγRIIaHR	FcγRIIaLR	FcγRIIaHR	FcγRIIaLR	FcγRIIb2	FcγRIIb2	FcγRIIb
MR7	IV.3	+ + +	+ + +	+ + +	+ + +	–	–	–
MA23	BAS62-11	–	–	–	–	–	–	–
MA72	KB61	±	±	±	±	+ + +	+ + +	+
MA73	2ZC115	–	–	–	–	+ + +	+ + +	+
MA128	FL18.26	+ +	+ +	+ +	+ +	+ +	+ +	+
MA126	CIKM5	+ +	+ + +	+	+ + +	–	–	–
MA179	AT10	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+
	IIIA5	+ +	+ +	–	–	+ +	–	–
	II8D2	+ +	–	–	–	+ +	–	–
	IA4	ND	ND	ND	ND	ND	ND	ND

Cells (see footnote to Table 1) were incubated with the mAb under saturating conditions at 4 °C (except for mAb MA73 where the cells were lysed before adding the antibody). The cells were subsequently lysed in modified RIPA buffer (10 mM Tris-HCl, pH 7.2; 1% w/v Triton-X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 150 mM NaCl; 5 mM Na-EDTA; 4 mM phenylmethylsulfonyl fluoride (PMSF); 1 TIU/ml aprotinin). The cell-free supernatant was subjected to Protein A + G-Sepharose (90 min, 4 °C). Bound immune complexes were eluted using sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After blotting on to nitrocellulose membranes, the various FcγRII isoforms were detected using mAb II8D2 (FcγRIIb) and IIIA5 (FcγRIIa + FcγRIIb). Bound mAb was detected after incubation with peroxidase-labelled goat anti-mouse IgG + IgM using the ECL chemiluminescence detection system (Amersham).

†ND, Not done. –, No reaction; ±, very weak reactivity; + to + + + indicate increasing levels of reactivity.

observed in FACS analyses, which seems to be mainly due to the lower affinity of this antibody for the FcγRIIa alloforms. The mAb MA73 (2ZC115), which is negative on intact cells (Table 1), specifically reacts with both FcγRIIb isoforms in cell extracts. Using a series of FcγRIIb2 mutants [2] lacking various numbers of amino acids from the carboxy-terminal end of the receptor we found that this mAb does not recognize FcγRIIb2 mutants lacking nine amino acids at the carboxy terminal end. In contrast, changing the Tyr273 residue into a Phe residue does not influence the reactivity of mAb MA73 (2ZC115).

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M8.3 Binding heterogeneity within the CD32 panel of mAb

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The CD32 molecule (FcγγRII) represents a 40-kDa low-affinity receptor for IgG, and is encoded by three genes, FcγRIIA, IIB, and IIC, all localized on chromosome 1q23–24. The transcripts derived from genes IIA (FcγRIIa) and IIB (FcγRIIb) differ both in their signal peptides and cytoplasmic domain-encoding regions, whereas extracellular and transmembrane encoding regions are ≈92 per cent homologous [1]. The FcγRIIC gene has been characterized as a result of a cross-over event between the IIA and IIB genes [2], and the transcript of gene IIC was found to be identical in its signal peptide and extracellular and transmembrane-encoding regions to FcγRIIb, whereas the cytoplasmic region-encoding domain was identical to that of FcγRIIa [3]. FcγRIIA is, furthermore, polymorphic and two allotypes have been defined that differ by a single amino acid (aa) at position 131 within the second Ig-like domain, where an arginine or histidine is found. These two allotypes differ in their ability to bind mouse (m) IgG1 complexes: FcγRIIa-R131 interacts effectively with mIgG1 (previous name: high-responder FcγRIIa), in contrast to FcγRIIa-H131 (previously: low-responder) [4]. Expression of the FcγRIIA gene is found on monocytes, neutrophils, and platelets [5], and evidence has been presented for (low-level) expression on B lymphocytes [6]. Additional diversity is found within the FcγRIIB subclass, which comprises three isoforms: FcγRIIb1, IIb2, and IIb3. The FcγRIIb2 isoform is identical to IIb1 except for the lack of a 19-aa insert in the cytoplasmic region (due to alternative splicing of the C1 exon). FcγRIIb3 is almost identical to IIb2, but lacks information for the putative signalase cleavage site, due to an alternatively spliced S2 exon [3]. The FcγRIIb1 and IIb2 transcripts were both found to be expressed on B lymphocytes [6].

In order to assess the reactivity of the CD32 Workshop panel monoclonal antibodies (mAb) with the different FcγRII molecules, we generated a panel of stable transfectants. Three different mouse cell lines were used for transfection, in order to avoid reactivity with endogenous human FcγRII molecules on cells, and to minimize other cell type-specific effects (for example, variation in glycosylation patterns). 3T6 fibroblasts were transfected with cDNAs encoding FcγRIIa-H131, IIa-R131, and FcγRIIb1* [4,7]. Of these, FcγRIIb1* is identical to FcγRIIb1 except for one aa difference at position 11 within the cytoplasmic tail, where a tyrosine (IIb1) is replaced by an aspartic acid (IIb1*). Moreover, these cDNAs, as well as FcγRIIb1 and IIb2 cDNAs, were expressed in a mFcγR-negative and surface IgG2a-positive mouse B-cell line IIA1.6 [6]. The third cell type we used for transfection was mouse T-cell line RMA-S, which binds anti-mouse FcγRII/III mAb 2.4G2 [8] and, thus, expresses endogenous mouse FcγR. These